INFECTION AND IMMUNITY, Sept. 1983, p. 1046–1055
0019-9567/83/091046-10$02.00/0

Vol. 41, No. 3

Antigenic Specificity and Heterogeneity of Lipopolysaccharides from Pyocin-Sensitive and -Resistant Strains of *Neisseria gonorrhoeae*

MARK C. CONNELLY† AND PETER Z. ALLEN*

Department of Microbiology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642

Received 28 March 1983/Accepted 6 June 1983

Homologous antisera were raised against lipopolysaccharides (LPSs) isolated from pyocin 103-sensitive JW31 strain *Neisseria gonorrhoeae* and its isogenic, pyocin-resistant variant, JW31R. Changes in immunochemical reactivity of LPS antigen associated with pyocin-resistance were examined by enzyme-linked immunosorbent assay, employing homologous and heterologous anti-LPS immune sera. The acquisition of pyocin 103 resistance is accompanied by a loss in LPS antigen reactivity with homologous anti-LPS. The variant LPS of pyocin 103-resistant mutants is immunogenic and displays a new, distinct antigenic specificity shared with other pyocin 103-resistant variant gonococcal strains. The acquisition of pyocin 103 resistance by JW31 strain gonococci is also accompanied by a striking loss of LPS cross-reactivity with antistreptococcal polysaccharide reagents having an antibody combining site specificity directed against the chemically defined lactose polymer from *Streptococcus faecalis* cell wall and pneumococcal type 14 capsular polysaccharide. When examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis, JW31 and JW31R LPSs show banding patterns characteristic of microheterogeneous, rough-type LPS devoid of O-side chains. Immunoblot transfer analysis of gel-separated gonococcal LPS antigens shows a difference in the pattern of antibody binding by homologous versus cross-reactive anti-LPS, which suggests a heterogeneity in the distribution of cross-reactive determinants among LPS molecules.

Pyocin 103, an R-type bacteriocin produced by *Pseudomonas aeruginosa* PA103, can inhibit the growth and exert bactericidal activity against *Neisseria gonorrhoeae* (16, 21). Lipopolysaccharide (LPS) has been identified as the cell surface component that serves as the pyocin receptor in sensitive strains of gonococci (7). Variation from pyocin sensitivity to pyocin resistance is accompanied by change in gonococcal LPS structure associated with loss of ability to function as a pyocin receptor (7), altered reactivity with lectins (7; M. C. Connelly and P. Z. Allen, Carbohydr. Res., in press) and serogroup-specific reagents (15), change in chemical composition (10; Connelly and Allen, in press), and alteration in sensitivity to normal serum and polymyxin B (10). In the present investigation, changes in gonococcal LPS antigen behavior associated with acquisition of pyocin 103 resistance were examined. Homologous antisera raised against gonococcal LPS isolated from pyocin 103-sensitive and -resistant variants were used in an enzyme-linked immunosorbent assay (ELISA) to demonstrate loss of parental type specificity and the acquisition of a new antigenic specificity by variant LPS associated with pyocin 103 resistance.

Antisera prepared against polysaccharide antigens of known structure provide immunochemical reagents of defined or restricted anti-carbohydrate specificity which can be exploited to detect structural changes in cross-reactive polysaccharide antigens of unknown structure (1). Heterologous antisera directed against a chemically defined lactose polymer from *Streptococcus faecalis* cell wall (18) and pneumococcal type 14 capsular polysaccharide (14) were also used in the ELISA to obtain additional immunochemical evidence for alteration of gonococcal LPS antigen structure associated with the appearance of pyocin resistance.

Heterogeneity and variation in sodium dodecyl sulfate (SDS)-polyacrylamide gel banding patterns of LPSs isolated from pyocin-resistant mutants of *Neisseria gonorrhoeae* have been described by Guymon et al. (10). More recently,

† Present address: Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824.

SDS-urea-polyacrylamide gel electrophoresis (PAGE) combined with a silver stain for detecting LPS in gels (23) has been employed by Tsai et al. (22) to obtain improved sensitivity and characterize banding patterns among meningococcal LPSs. Both SDS-PAGE and SDS-urea-PAGE, combined with silver staining, were used in the present study to characterize LPS antigen isolated from the pyocin 103-sensitive JW31 strain of *N. gonorrhoeae* and its isogenic, pyocin-resistant variant, JW31R. Immunoblot analysis of bands separated in polyacrylamide gels was used to examine fractions of transferred gonococcal LPS antigen for heterogeneity in the ability to bind homologous or cross-reactive anti-LPS.

MATERIALS AND METHODS

Organisms. Pyocin 103-sensitive *N. gonorrhoeae* strains JW31 and RUG40 and their pyocin-resistant variants JW31R, RUG40R-11, and RUG40R-16, grown as T1 or T4 colony types, have been described previously (2, 7). Gonococci were grown in the liquid GCP medium described by Connelly et al. (7) or Catlin's chemically defined medium (6). Cultures of pyocin 103-resistant strain JW31R, used to isolate JW31R LPS, were grown in the continuous presence of excess pyocin 103 to eliminate pyocin-sensitive revertants. *S. faecalis* strain N, provided by J. H. Pazur, was grown in Todd-Hewitt broth, and killed cultures were used to prepare the vaccine (17).

Antigens and antisera. LPSs were isolated from gonococci by hot phenol extraction (20). Purified gonococcal LPS was essentially free of protein contamination. Solutions (1.0 mg/ml) of gonococcal LPS in 10 mM phosphate-buffered saline (pH 7.2) gave an optical density at 277 nm of <0.010 and showed no Coomassie brilliant blue-stained bands when applied to SDS-PAGE or SDS-urea-PAGE gels in amounts up to 5 μg. LPSs from *Salmonella typhimurium*, *Salmonella minnesota* Re595, and *Escherichia coli* O55:B5, used as gel reference markers, were obtained from Calbiochem-Behring (San Diego, Calif.). Lipid A was recovered from isolated JW31 LPS after mild acetic acid treatment as described by Perry et al. (20). Saline solutions of isolated, purified JW31 or JW31R LPS used as antigen were mixed with an equal volume of Freund complete or incomplete adjuvant (Difco Laboratories, Detroit, Mich.) to give emulsions containing about 1 mg of LPS per ml. Rabbits (female, New Zealand White) initially received a total of 1 mg of LPS in Freund complete adjuvant, injected subcutaneously into multiple sites. Booster injections were given at 6- to 8-week intervals by subcutaneous injection of a total of 0.5 mg of LPS in incomplete Freund adjuvant. Bleedings were taken 2 to 3 weeks after each series of injections to provide antisera obtained after each of eight successive courses of booster stimulation. Immune sera were designated −1C through −8C to denote bleedings taken after each successive course of booster injection. R2 antisera (bleedings 1C through −8C) were obtained from rabbit R2 immunized with JW31 LPS. R1 antisera (bleedings −1C through 8C) were obtained from rabbit R1 injected with JW31R variant LPS.

Gonococcal suspensions (RUG40 T1, RUG40 T4, RUG40R-11, RUG40R-16) used as vaccines were grown in chemically defined, liquid medium. Log-phase cultures, killed by the addition of Formalin (2%, vol/vol) were washed three times by centrifugation in saline containing 0.25% phenol and then suspended in saline containing 0.25% (wt/vol) phenol. The volume of washed, killed, gonococcal vaccine suspensions was adjusted to give an OD_{650} of 1.0 (approximately 2.6×10^9 bacteria per ml). Rabbits received 1 ml of vaccine per week, given subcutaneously as four 0.25-ml doses. Animals were bled 2 weeks after having received a total of 3 ml of vaccine. After a 4-week rest period, rabbits were given a series of booster injections similar to those described for primary immunization. Anti-*S. faecalis* immune sera were raised in rabbits injected with a streptococcal vaccine prepared and administered as described in detail by Pazur et al. (17). Immune serum from a single rabbit (R1) was obtained from bleedings taken after each of seven courses of injections with streptococcal vaccine and was used in the ELISA to examine cross-reaction with gonococcal LPS. In immunodiffusion, rabbit anti-*S. faecalis* sera produced only one band of precipitation with crude streptococcal cell-wall extract or purified cell-wall lactose polymer provided by J. H. Pazur. Type 14 antipneumococcal rabbit serum (Lederle), provided by G. Schiffman, was used at 1/1,500 dilution in the ELISA. All sera were preserved by the addition of 5% (wt/vol) phenol and 1% merthiolate (Eli Lilly & Co., Indianapolis, Ind.) to give final concentrations of 0.25% and 0.01%, respectively.

Affinity purification of goat anti-rabbit IgG. Anti-rabbit immunoglobulin G (IgG) serum (G1-1C) was raised by immunizing a goat with isolated, rabbit IgG purified by ion-exchange column chromatography on diethylaminoethyl-cellulose. The goat received 25 mg of IgG, injected subcutaneously as alum-precipitated antigen (12). Immunodiffusion and immunoelectrophoresis showed IgG to be the only rabbit serum component reactive with immune goat serum. Affinity purification of goat anti-rabbit IgG was achieved by immunoadsorption on a water-insoluble polymer of normal rabbit serum prepared by the ethyl chloroformate method of Avrameas and Ternynck (4). A 4-ml pellet of thoroughly washed normal rabbit serum polymer was suspended in 20 ml of goat anti-rabbit IgG and incubated for 60 min at 37°C and then for 60 min at 4°C. The immunoadsorbent was then sedimented by centrifugation and washed repeatedly in 0.05 M phosphate buffered-saline (PBS), pH 7.2, until supernatant washings were free of OD_{280}. Bound antibody was eluted from the immunoadsorbent with 0.05 M glycine-hydrochloride buffer (pH 2.5) at 4°C. Eluates were neutralized with sodium borate and then concentrated by ultrafiltration. Isolated, affinity purified, goat anti-rabbit IgG was stored frozen at −20°C in 1-ml samples containing approximately 1.28 mg of antibody per ml. The antibody content of solutions in 0.025 M PBS (pH 7.2) was estimated spectrophotometrically by using a specific extinction coefficient of 0.0090 OD_{277} (1-cm light path) per μg N per ml for goat IgG immunoglobulin and a protein nitrogen content of 16% to convert nitrogen weight to protein.

Coupling alkaline phosphatase to antibody. Alkaline

phosphatase was covalently coupled to affinity-purified goat anti-rabbit IgG by the glutaraldehyde cross-linking method of Engvall and Perlmann (8). Approximately 1 mg of alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo., type VII) was mixed with 1 ml (1.28 mg of antibody) of goat anti-rabbit IgG and dialyzed against PBS overnight at 4°C. Glutaraldehyde (50%, wt/wt) was diluted 1/10 in PBS and slowly added to the antibody-enzyme mixture until a final glutaraldehyde concentration of 0.2% (wt/vol) was reached. The reaction mixture was incubated for 2 h at 23°C with occasional stirring. Antibody-enzyme conjugates were dialyzed overnight at 4°C in PBS, followed by several changes of 0.05 M Tris buffer (pH 8.0) containing 1 mM $MgCl_2$ and 0.025% (wt/vol) sodium azide. Enzyme-coupled antibody was stored at 4°C with 5% (wt/vol) bovine serum albumin (BSA).

ELISA assay conditions. The ELISA was performed essentially as described by Apicella and Gagliardi (3) with 96-well, U-bottomed microtiter ELISA plates (Dynatech Laboratories Inc., Alexandria, Va.). Solutions of antigen (0.1 ml) in 0.05 M carbonate-bicarbonate buffer containing 0.02 M $MgCl_2$ (pH 9.6) were added to microtiter wells and incubated for 3 h at 37°C and then overnight at 4°C. After antigen binding, and between each subsequent addition of reagents, plates were washed three times in BSA-buffer containing 5% (wt/vol) BSA, 0.01 M PBS, 0.02 M $MgCl_2$, and 0.025% (wt/vol) sodium azide. The BSA-buffer solution was prepared in advance and prefiltered through 0.3-μm filters (Millipore Corp., Bedford, Mass.) before storage at 4°C. After plates were washed, 0.1 ml of rabbit antiserum diluted in BSA-buffer was added to wells and incubated for 6 to 8 h at 23°C. All incubations with antisera were carried out in humidified chambers. Plates were again washed in BSA-buffer. Alkaline phosphatase-coupled goat anti-rabbit IgG, diluted 1/100 in BSA-buffer, was added to wells (0.1 ml), and plates were incubated at 23°C overnight. After washing, 0.1 ml of *p*-nitrophenyl phosphate (1 mg/ml; Sigma) in 0.05 M carbonate buffer (pH 9.8) containing 1 mM $MgCl_2$ was added to wells, and plates were incubated for 60 min at 23°C. The enzyme reaction was stopped by the addition of 0.1 ml of 2 N NaOH to each well, and the OD_{410} was read by using a Dynatech Microelisa Minireader MR 590.

The effect of variation in JW31 and JW31R LPS antigen-coating concentration on the amount of antibody bound to microtiter plates was examined over a range of concentrations varying from 0.1 to 50 μg of LPS per ml. Homologous and cross-reactive antibody bound reached a maximum at 10 μg/ml and remained constant over the range 10 to 50 μg/ml; therefore, a standard antigen-coating concentration of 15 μg/ml was used in ELISA assays. The levels of antibody bound determined by ELISA were recorded as the arithmetic mean and standard error of the mean of three or more separate determinations.

PAGE. Polyacrylamide gels were prepared essentially as described by Laemmli (13). Separating gels (11.2 by 14.6 by 0.12 cm) were prepared containing 16.5% (wt/vol) acrylamide, 0.44% (wt/vol) *N,N'*-methylenebisacrylamide, 0.373 M Tris buffer (pH 8.9), 0.1% (wt/vol) SDS (Bio-Rad Laboratories, Richmond, Calif.; electrophoresis purity), 0.1% (wt/vol) ammonium persulfate, and 0.05% tetramethylenediamine (Eastman Kodak, Co., Rochester, N.Y.). After polymerization, the upper surface of the gel was washed with water and blotted dry; a stacking gel of 5% acrylamide, 0.13% *N,N'*-methylenebisacrylamide, 0.124 M Tris buffer (pH 6.8), 0.1% ammonium persulfate, and 0.05% tetramethylenediamine was layered on top.

SDS–4 M urea–PAGE. A stock solution of 4 M urea containing 0.01% (vol/vol) ethanolamine was prepared for SDS-urea-PAGE. To make 4 M urea-polyacrylamide gels, all reagents were freshly prepared as described for SDS-PAGE, except 4 M urea stock solution was substituted for deionized water. The sample buffer, prepared by the method of Tsai and Frasch (23), contained 0.1 M Tris buffer (pH 6.8), 2% (wt/vol) SDS, 20% (wt/vol) sucrose, 1% (vol/vol) 2-mercaptoethanol, and 0.005% (wt/vol) bromphenol blue. Equal volumes of sample buffer and sample were mixed, heated for 3 min at 100°C, and loaded onto the gel. Electrophoresis for both SDS-PAGE and SDS-Urea-PAGE was done with pH 8.3 buffer containing 0.025 M Tris-hydrochloride, 0.192 M glycine, and 0.01% (wt/vol) SDS (13). A potential of 60 V was maintained across the gel until the bromphenol blue dye front had traveled to within 3 mm of the bottom of the separating gel.

Silver staining of polyacrylamide gels. The silver stain procedure of Tsai and Frasch (23) was used to stain gels. Gels were fixed in three 1-liter changes of 40% (vol/vol) ethanol–5% (vol/vol) acetic acid and then treated for 5 min with 0.78% (wt/vol) periodic acid fixing solution. Treated gels were washed three times in deionized water (1 liter, 15 min per wash), then incubated for 10 min with an ammoniacal silver solution containing 1 g of $AgNO_3$, 28 ml of 0.1 N NaOH, 2 ml of concentrated NH_4OH, and 120 ml of deionized water. Gels were again washed three times in deionized water. To visualize silver-stained bands, a solution containing 50 mg of citric acid and 0.5 ml of 37% (wt/vol) formaldehyde solution was prepared in 200 ml of deionized water and added to gels, and gels were incubated until the desired density of stain was reached (1 to 2 min).

Immunoblot transfer of LPS. Immunoblot electrophoretic transfer of LPS was done by using a procedure modified from that of Burnette (5). Immediately after electrophoresis, the stacking gel was removed, and the lower right corner of the separating gel was notched for orientation. A TE 42 Transphor electrophoresis transfer cassette and electrophoresis unit (Hoefer Scientific Instruments) was used for electrophoretic transfer of LPS from polyacrylamide gels to nitrocellulose filters (BA 85, 0.45 μm; Schleicher & Schuell Co., Keene, N.H.). Electrophoresis transfer buffer (pH 8.2) consisted of a solution of 25 mM Tris base made 192 mM with glycine. Polyacrylamide gels were loaded into the electrophoresis unit with the nitrocellulose filter on the anode side of the gel and electrophoresed for 1 h at 400 mA. After transfer, all nitrocellulose filters were stored overnight at 4°C. Filters were then incubated for 1 h at 37°C with PBS buffer containing 3% (wt/vol) BSA to saturate all remaining binding sites. This step was done with constant shaking, as were all subsequent steps. Nitrocellulose filters were then cut lengthwise into strips 2 cm wide containing each lane of sample and placed into separate 50-ml disposable centrifuge tubes. Individual transfer strips were incubated for 90 min at 23°C

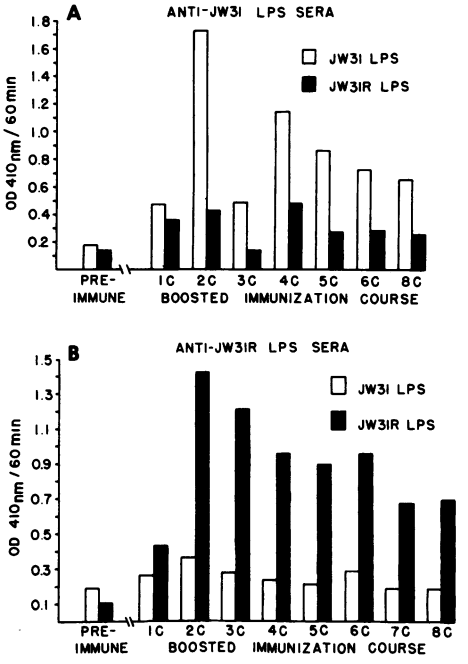

FIG. 1. Assay by ELISA for binding to JW31 and JW31R LPS antigen by immune sera obtained from rabbits after each of eight successive courses of immunization with LPS. (A) Anti-JW31 (rabbit 2) immune sera assayed at 1/250 dilution. (B) Anti-JW31R (rabbit 1) immune sera assayed at 1/500 dilution.

with antiserum (10 ml) diluted 1/10 in PBS buffer containing 3% BSA and 0.05% (vol/vol) Tween 80 and then washed two times in 40 ml of PBS and two times in 40 ml of PBS containing 0.05% Tween 80 (20 min per wash). All strips then received 10 ml of ^{125}I-protein A (New England Nuclear Corp., Boston, Mass.), in PBS–BSA–Tween 80 buffer (approximately 40,000 cpm/min) and were incubated for 90 min at 23°C. Nitrocellulose filter strips were washed as described above, blotted dry, and autoradiographed overnight at −70°C with Kodak AR film contained in an X-Omatic cassette equipped with intensifying screens (Eastman Kodak Co.). Efforts to stain nitrocellulose strips or PAGE gels directly with silver after electrophoetic blot transfer gave uniform blackening of the entire strip or gel and were therefore unsatisfactory for use as controls.

RESULTS

Response to immunization with JW31 and JW31R LPS. Sera obtained from rabbits immunized with purified gonococcal LPS after primary (1C) and repeated booster stimulation (2C through 8C) were examined for antibody activity by the ELISA (Fig. 1). Both JW31 and JW31R gonococcal LPSs are immunogenic and give rise to a specific anti-LPS response. Primary and secondary immunization resulted in a two- to eightfold increase in reactivity with homologous antigen above levels given by pre-immunization bleedings. An antibody specificity directed against the antigen used for immunization is evident from a comparison of levels of serum reactivity shown by ELISA with homologous and heterologous antigens. Sera raised in rabbit R2, injected with JW31 LPS, showed a greater reactivity with homologous JW31 than with JW31R variant LPS (Fig. 1A). Conversely, rabbit R1, injected with JW31R variant LPS, produced antisera that showed significantly greater reactivity with JW31R variant LPS (Fig. 1B). Despite repeated booster injections (Fig. 1A and B, bleedings 3C through 8C), an anamnestic rise in anti-LPS levels was not obtained; rather, antibody levels showed a gradual decline during continued or prolonged immunization.

ELISA with heterologous antigonococcal sera. Antisera raised against Formalin-killed cell suspensions of RUG40 strain gonococci grown as T1 or T4 colony types and antisera raised against pyocin 103-resistant RUG40 variants RUG40R-11 and RUG40R-16, as well as antisera against isolated RUG40 (T1) LPS, were assayed for their ability to cross-react with JW31 and JW31R LPS. As illustrated in Fig. 2, antisera raised against pyocin 103 sensitive RUG40 strain vaccines or isolated RUG40 LPS showed a high degree of reactivity with wild-type JW31 LPS, whereas the same sera showed little or no reactivity with JW31R variant LPS. Conversely, antisera raised against pyocin 103-resistant vari-

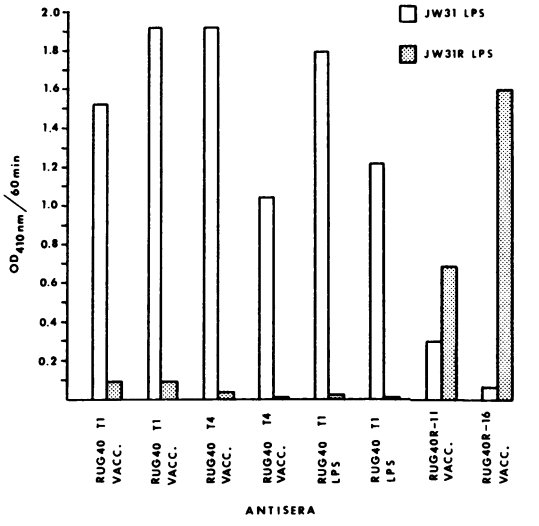

FIG. 2. ELISA cross-reactivity of anti-RUG40 immune sera with JW31 and JW31R LPS antigen. JW31 and JW31R LPS antigens (15 μg/ml) were reacted with rabbit antisera to the following: RUG40, pyocin-sensitive whole organism vaccine with either T1 or T4 colony morphology; isolated RUG40 LPS; vaccines of RUG40R pyocin-resistant variants RUG40R-11 and RUG40R-16.

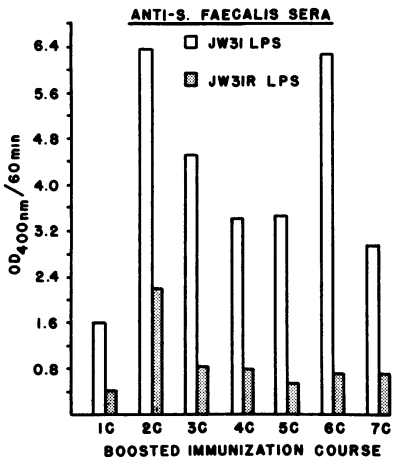

FIG. 3. Assays by ELISA of JW31 and JW31R gonococcal LPS antigen reactivity with rabbit anti-*S. faecalis* immune sera. Rabbit antisera 1C through 8C, obtained after each of eight successive courses of injection with streptococcal vaccine.

ant gonococcal vaccines RUG40R-11 and RUG40R-16 showed a 2- to 17-fold greater reactivity with JW31R variant LPS than with JW31 LPS.

ELISA with anti-S. faecalis sera. Sera obtained from a single rabbit after each of a series of successive courses of booster injections with *S. faecalis* vaccine were examined by ELISA for cross-reactivity with JW31 and JW31R LPSs. As illustrated in Fig. 3, although all bleedings showed binding to both antigens, anti-streptococcal sera showed a three to ninefold greater binding activity when assayed with JW31 LPS than with JW31R variant LPS.

ELISA with rabbit anti-pneumococcal type 14 serum. Figure 4 shows the results of the ELISA of rabbit anti-pneumococcal type 14 capsular polysaccharide antibody binding to microtiter plates at various coating concentrations of gonococcal LPS. Anti-pneumococcal type 14 antibody showed high binding activity with all coating concentrations of JW31 LPS, whereas JW31R variant LPS gave significantly less binding and showed only 1/4 to 1/15 the reactivity of JW31 LPS.

SDS-PAGE with silver staining. LPSs from strain JW31, JW31R, and RUG40 gonococci were examined by SDS-PAGE (Fig. 5A). Smooth (S-type) LPS from *S. typhimurium* and *E. coli* O55:B5 and rough (R-type) LPS from *S. minnesota* Re595 lacking O-side chains were included as reference standards. *S. typhimurium* S-type LPS gave a series of bands (Fig. 5A) corresponding to molecules with increasing numbers of O-side chain repeating units attached to the core (9, 11, 19). The bulk of R-type *S. minnesota* Re595 LPS (Fig. 5A) migrated further than *s. typhimurium* LPS, although a small amount of LPS having O-side chains was also detected in the R-type reference standard. As shown in Fig. 5A, LPSs from gonococcal strains RUG40, JW31, and JW31R yielded only low-molecular-weight components and showed no banding pattern indicative of O-side chain structure. Although a heterogeneous array of O-side chain lengths was not found among gonococcal LPS preparations, limited heterogeneity among low-molecular-weight components was clearly evident since JW31R variant LPS gave one broad, diffuse band (Fig. 5A), and JW31 variant LPS gave two bands (Fig. 5A), whereas RUG40 variant LPS gave two broad, poorly resolved bands with 1- and 2-μg samples (Fig. 5A). RUG40 LPS showed a third faint band when only a 0.5-μg sample was applied to the gel (Fig. 5A).

SDS-urea-PAGE with silver staining. The addition of 4 M urea to SDS-polyacrylamide gels markedly improved LPS band resolution (Fig. 5B). Distinct band resolution was particularly evident with low-molecular-weight LPS species; compare SDS-PAGE without urea (Fig. 5A) with corresponding samples run in 4 M urea (Fig. 5B). SDS-urea-PAGE revealed more gonococcal LPS heterogeneity than did SDS-PAGE. JW31 LPS gave six bands in urea gels (Fig. 5B) compared with two bands obtained without urea (Fig. 5A). JW31R LPS gave five distinct, low-

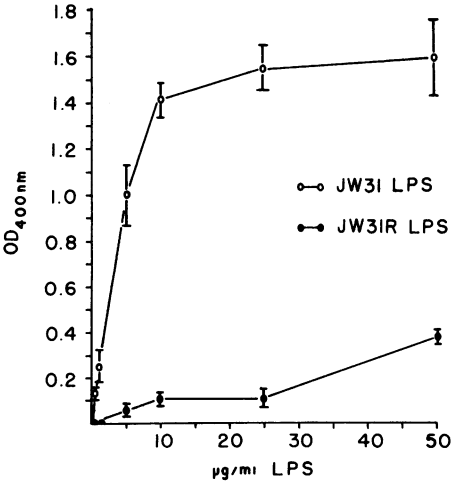

FIG. 4. ELISA of rabbit anti-*S. pneumoniae* type 14 antibody binding to JW31 and JW31R gonococcal LPS antigen. Various concentrations of JW31 or JW31R LPS were reacted with pneumococcal type 14 antiserum at 1/1,500 dilution. Each value represents the arithmetic mean (± standard error of mean) of three separate determinations after subtracting values obtained with normal rabbit serum.

molecular-weight bands in urea gels (Fig. 5B), whereas only a single, intense, broad band was obtained in gels without urea (Fig. 5A). In addition, when large amounts of sample (2 μg) were applied, a faint-staining, slowly migrating, higher-molecular-weight component in both JW31 and JW31R gonococcal LPS preparations was clearly seen in urea gels and was not evident in gels without urea.

Immunoblot transfer of JW31 LPS from SDS-PAGE gels. JW31 LPS antigen and its isolated lipid A moiety were subjected to SDS-PAGE and then examined for the ability to bind antibody after blot transfer to nitrocellulose, exposure to antibody, treatment with radiolabeled protein A, and autoradiography to locate bound antibody (Fig. 6). Individual membrane strips were treated with (i) rabbit R2 preimmunization serum, (ii) anti-JW31 LPS serum R2-2C, (iii) anti-JW31R LPS serum R1-2C. Binding of anti-JW31 LPS antibody (Fig. 6, lane 2) gave two bands; one broad, intense upper band and a narrower, lower band denoting a smaller antigen species. Both anti-JW31R and rabbit R2 preimmunization serum (Fig. 6) showed binding, although binding by pre-immunization rabbit R2 serum was relatively less intense. Binding by these two sera appeared restricted, however, to selective portions of the transferred antigen detected by homologous antibody (Fig. 6; compare lane 2 with lanes 1 and 3). Binding to the lipid A moiety of JW31 LPS by anti-JW31 immune serum was spread over a broad range of the transfer strip, showing that isolated lipid A does not form discrete bands in PAGE. That binding of anti-JW31 antibody to lipid A is specific is shown by the failure of preimmune rabbit R2 serum and anti-JW31R LPS immune serum to produce any etectable bands. The absence of nonspecific protein A binding to lipid A is evident from control lane 4 in Fig. 6, which fails to show any band after autoradiography.

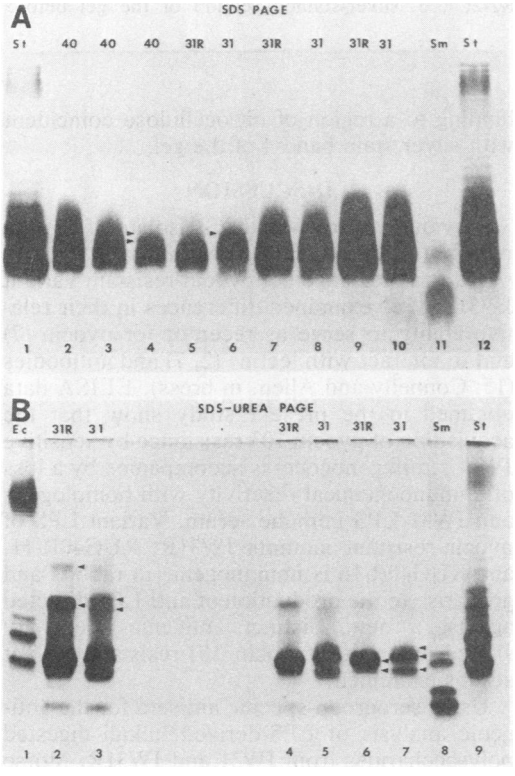

FIG. 5. (A) Silver stain of SDS-PAGE-separated LPS. Lanes: 1, *S. typhimurium* LPS, 7.5 μg; 2, RUG40 LPS, 2.0 μg; 3, RUG40 LPS, 1.0 μg; 4, RUG40 LPS, 0.5 μg; 5, JW31R LPS, 0.5 μg; 6, JW31 LPS, 0.5 μg; 7, JW31R LPS, 1.0 μg; 8, JW31 LPS, 1.0 μg; 9, JW31R LPS, 2.0 μg; 10, JW31 LPS, 2.0 μg; 11, *S. minnesota* Re595 LPS, 1.0 μg; 12, *S. typhimurium* LPS, 7.5 μg. (B) Silver stain of SDS-urea-PAGE-separated LPS. Lanes: 1, *E. coli* O55:B5 LPS, 5.0 μg; 2, JW31R LPS, 2.0 μg; 3, JW31 LPS, 2.0 μg; 4, JW31R LPS, 1.0 μg; 5, JW31 LPS, 1.0 μg; 6, JW31R LPS, 0.5 μg; 7, JW31 LPS, 0.5 μg; 8, *S. minnesota* Re595 LPS, 1.0 μg; 9, *S. typhimurium* LPS, 5.0 μg.

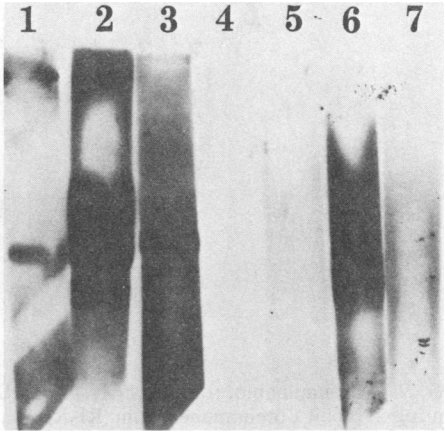

FIG. 6 Immunoblot transfer analysis of SDS-PAGE-separated JW31 LPS and JW31 lipid A. Lanes: 1, JW31 LPS reacted with rabbit 2 preimmune serum; 2, JW31 LPS reacted with anti-JW31 LPS serum (R2-2C); 3, JW31 LPS reacted with anti-JW31R LPS serum (R1-2C); 4, JW31 lipid A reacted with ^{125}I-protein A alone; 5, JW31 lipid A reacted with rabbit 2 preimmune serum; 6, JW31 lipid A reacted with anti-JW31 LPS serum (R2-2C); 7, JW31 lipid A reacted with anti-JW31R LPS serum (R1-2C).

Immunoblot transfer of JW31 LPS from SDS-urea-PAGE. Antibody binding to JW31 gonococcal LPS separated by SDS-urea-PAGE is shown in Fig. 7A. LPS (1 μg) antigen was applied to a series of six gel slots and then subjected to SDS-urea-PAGE. One vertical end lane was sliced from the gel and silver stained before electro-

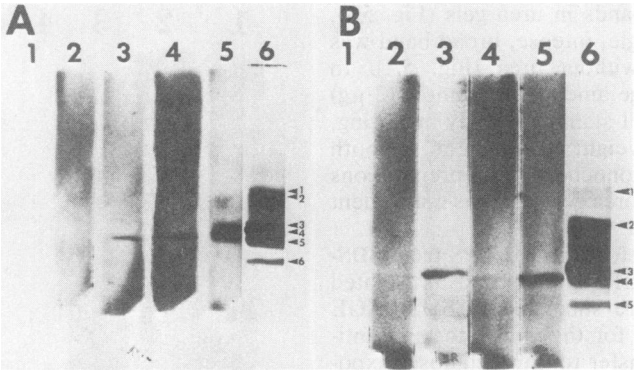

FIG. 7. (A) Immunoblot transfer analysis with SDS-urea-PAGE-separated JW31 LPS. Lanes: 1, ^{125}I-protein A control; 2, rabbit 1 preimmune serum (R1-X); 3, anti-JW31R LPS serum (R1-2C); 4, rabbit 2 preimmune serum (R2-X); 5, anti-JW31 LPS serum (R2-2C); 6, Silver-stained section of the gel before electrophoretic transfer of JW31 LPS. (B) Immunoblot transfer analysis with SDS-urea-PAGE-separated JW31R LPS. Lanes: 1, ^{125}I-protein A control; 2, rabbit 1 preimmune serum (R1-X); 3, anti-JW31R LPS serum (R1-2C); 4, rabbit 2 preimmune serum (R2-X); 5, anti-JW31 LPS serum (R2-2C); 6, silver-stained section of the gel before electrophoretic transfer of JW31R LPS.

phoretic blot transfer of the parent gel slab. As shown in Fig. 7A, lane 6, six separate, discrete, silver-stained bands were obtained from the gel track of antigen. In contrast to the banding pattern developed by silver stain, autoradiography after anti-JW31 antibody binding (Fig. 7A, lane 5) produced only a single broad band limited to a region of gel colinear with silver-stained bands 4 and 5 and perhaps also band 3. Similar to results obtained in SDS-PAGE without urea, preimmune rabbit R2 serum, which contains a naturally occurring cross-reactive anti-LPS and anti-JW31R variant LPS immune serum R1-2C show antibody binding restricted to a gel region of antigen coincident with silver-stained band 5. Binding of antibody to regions of gel corresponding to silver-stained bands 1, 2, and 6 was not detected even after prolonged autoradiography. Control lanes (Fig. 7A), which included ^{125}I-protein A and preimmune serum from rabbit R1 lacking naturally occurring cross-reactive anti-LPS, showed no LPS binding.

Immunoblot transfer of JW31R LPS from SDS-urea-PAGE. LPS from the JW31R variant strain of gonococcus was subjected to SDS-urea-PAGE, and the gel slab was treated as described above for type JW31 LPS. Direct silver staining of a reference gel lane is shown in Fig. 7B, lane 6, with five reference silver stain bands indicated. After blot transfer from the parent gel, anti-JW31R immune serum R1-2C and anti-JW31 immune serum R2-2C each showed a single narrow region of antibody binding coincident with silver stain band 4 of the gel. Although preimmune serum from rabbit R1 showed no binding to JW31R variant LPS, preimmune rabbit 2 serum showed very faint, but detectable, binding to a region of nitrocellulose coincident with silver stain band 4 of the gel.

DISCUSSION

Previous studies with LPSs isolated from the pyocin 103-sensitive JW31 strain of *N. gonorrhoeae* and its isogenic, pyocin-resistant variant JW31R have examined differences in their relative ability to serve as receptor for pyocin (7) and to interact with lectins (2, 7) and antibodies (15; Connelly and Allen, in press). ELISA data obtained in the present study show that the acquisition of pyocin 103 resistance by sensitive JW31 strain gonococci is accompanied by a loss of immunochemical reactivity with homologous anti-JW31 LPS immune serum. Variant LPS of pyocin-resistant mutants JW31R, RUG40R-11, and RUG40R-16 is immunogenic in rabbits and gives rise to the production of anti-LPS directed against a new, distinct antigenic specificity shared among the pyocin 103-resistant variant strains examined.

Using serogroup-specific antisera for the antigenic analysis of LPS-derived, alkali digested polysaccharides from JW31 and JW31R, Morse and Apicella (15) showed JW31 to belong to serogroup Gc_4. In addition, JW31 was found to possess both the "variable common" and "universal" antigenic specificities (3, 15). In contrast, JW31R variant LPS was devoid of serogroup and variable common antigenic reactivity; however, a portion of the universal common antigenic specificity was retained (15). The low levels of cross-reactivity found by ELISA in the present study between JW31 antigen and anti-JW31R as well as between JW31R LPS antigen and anti-JW31 immune serum is in accord with

the results of others and demonstrates further the antigenically distinct nature of gonococcal variant LPS employing specific homologous antisera. Employing Gc serogroup-specific reagents provided by Michael Apicella (3), RUG40 LPS has been found to possess antigenically distinct Gc_2 serogroup-specific determinants. The presence of common antigenic specificities shared by gonococcal strains JW31 and RUG40 is indicated, however, by the strong cross-reactivity exhibited by JW31 LPS antigen in the ELISA with antisera raised against RUG40 cell vaccines or isolated RUG40 LPS. The weak or barely detectable cross-reaction of RUG40 antisera with JW31R variant LPS antigen in the ELISA stands in marked contrast to their extensive cross-reactivity with the JW31 LPS antigen. Conversely, antisera raised against pyocin 103-resistant RUG40R-11 and RUG40R-16 variants show extensive cross-reaction with JW31R LPS, but little or no reactivity with JW31 LPS. The strong cross-reactions exhibited by JW31R LPS antigen with antisera raised against RUG40R-11 and RUG40R-16 suggest that these variants share antigenic determinants either not found or sequestered in the LPS of isogenic, pyocin-sensitive RUG40. The binding of anti-RUG40R-11 and anti-RUG40R-16 antibodies to JW31R LPS antigen demonstrated by ELISA could be mediated by multiple shared antigenic determinants or by a single common determinant.

The acquisition of pyocin 103 resistance by JW31 strain gonococci is also accompanied by a striking loss of LPS cross-reactivity with anti-streptococcal antibodies having a combining site specificity directed, at least in part, against lactose (anti-*S. faecalis*) or *N*-acetyl lactosamine (anti-*S. pneumoniae* type 14). The detailed chemical fine structure of gonococcal LPS is not known, nor is the structural basis for differences in their immunochemical behavior yet established. Whether the appearance of new antigenic determinants and the disappearance of cross-reactivity with anti-lactose and anti-*N*-acetyllactosamine reagents can be ascribed chemically to a simple loss of sugar units, anomerization of glycosidic bonds or the sequestration of antigenic determinants by the addition of new substituent groups to LPS core structure requires chemical study and is not established by the present data.

SDS-Page has proven to be an effective means for demonstrating bacterial LPS heterogeneity. Smooth-type enterobacterial LPS shows a periodic array of bands corresponding to molecules which differ in the number of O-side chain repeating units attached to the core (9, 11, 19). Salmonella LPS core contains 11 sugar residues, and the O-side chain of *S. typhimurium* is composed of a five-sugar repeating unit. Analysis of RUG40, JW31, and JW31R gonococcal LPSs by SDS-PAGE gave no banding pattern indicative of O-side chain structure. In SDS-PAGE, JW31 and JW31R LPSs migrate to a position between the core (11 sugars long) and the core plus one unit of O-side chain (16 sugars long). Thus, the apparent size of JW31 and JW31R LPS, as estimated by SDS-PAGE, is between 11 to 16 sugars long plus the contribution of the lipid A moiety.

LPS isolated from several pyocin-resistant mutants derived from strain FA19 *N. gonorrhoeae* have been analyzed chemically and characterized by Guymon et al. (10), who found variant gonococcal LPS to migrate as one or two distinct molecular species. More recently, employing SDS-urea-PAGE combined with sensitive silver staining, microheterogeneity among isolated R-type LPSs lacking O-side chains has been demonstrated for eight immunotype LPSs of *N. meningitidis* (22). Preparations of meningococcal LPS were shown by Tsai et al. (22) to contain two predominant components with molecular weights equivalent to those of rough enterobacterial LPSs having complete and incomplete cores. While the major meningococcal LPS component was estimated to be in the molecular weight range of 4,200 to 5,000, a few minor, higher-molecular-weight components were also detected. Incorporation of 4 M urea into polyacrylamide gels, as suggested by Tsai et al. (22), was found to improve resolution of gonococcal LPS bands. Several bands were obtained for both JW31 and JW31R in SDS-urea-PAGE not present in SDS-PAGE. The heaviest silver staining components of JW31R LPS formed a doublet, whereas JW31 LPS formed a triplet. A striking difference between results obtained with and without urea is the presence of one or two additional minor, high-molecular-weight components in SDS-urea-PAGE, best demonstrated with a large sample size. Although these bands could represent a high-molecular-weight LPS species detected because of improved resolution, they probably arise from incompletely disaggregated LPS. Lipid A-free carbohydrate chains obtained from JW31 and JW31R LPS after mild acid hydrolysis show both strains to possess R-type LPS, devoid of O-side chains, with cores estimated by gel filtration to have molecular weights of about 1,800 (Connelly and Allen, in press). The structural basis for the observed microheterogeneity among gonococcal LPS molecules is not known. Whether banding in SDS-urea-PAGE represents incomplete or altered core structure as shown for meningococcal LPS (22) or whether qualitative or quantitative differences in amide- or acyl-linked fatty acids within the lipid A moiety may also contribute is not known.

To examine whether bands obtained in PAGE are associated with differences in antibody reactivity, immunoblot analysis of SDS-PAGE-separated LPS and its lipid A moiety was carried out. Anti-JW31 serum showed binding to two discrete regions of homologous LPS antigen after transfer to nitrocellulose and uniform binding to its transferred lipid A moiety. Although antibody to JW31R variant LPS and preimmune rabbit 2 serum show binding to JW31 LPS, this binding is confined entirely to the lowest portion of the higher-molecular-weight antigen species identified by homologous antibody. The lower-molecular-weight band that transfers to nitrocellulose and binds homologous antibody fails to bind heterologous cross-reactive antibody. Thus, after electrophoretic transfer, companion strips of the same transferred band show an internal difference in the distribution pattern of bound antibody. This restricted pattern in antibody binding indicates a heterogeneity in antigen reactivity and suggests that binding observed with heterologous sera may be mediated by a particular subpopulation of antigen molecules. The finding that heterologous sera bind to intact JW31 LPS antigen, yet show no binding to lipid A, indicates that lipid A binding by homologous anti-JW31 serum is specific and suggests that antigenic determinants involved in the binding of cross-reactive antibody reside outside the lipid A region of the LPS molecule. Although reference gel tracks of SDS-urea-PAGE-separated JW31 antigen yield six bands with silver stain, only two or perhaps three of these bands appear to bind homologous antibody after transfer to nitrocellulose. Similarly, blot transfer of JW31R LPS antigen from urea gels showed binding to only one of five silver-stained gel bands. The apparent failure of a particular silver-stained gel band to bind antibody after a companion gel track has been transferred to nitrocellulose could arise from a failure to bind antibody due to inappropriate antigen structure. However, such an interpretation requires the further demonstration that nonreactive bands do, in fact, transfer out of the gel, bind to nitrocellulose, and withstand subsequent washing. Thus, the finding that several components of gonococcal LPS demonstrated by SDS-PAGE and silver stain fail to bind antibody after blot transfer may not be unequivocally due to differences in antigen reactivity. Although a comparison of stained gels with immunoblot transfer strips presents difficulties, an internal comparison of companion nitrocellulose strips cut from the same blot transfer avoids these limitations. In agreement with data obtained in the absence of urea, immunoblot transfers of JW31 LPS antigen from SDS-urea-PAGE gels show an internal difference in the distribution pattern of bound homologous versus heterologous antibody among companion filter strips indicative of antigen heterogeneity.

ACKNOWLEDGMENTS

This research was supported by the New York State Health Planning Commission, Health Research Council grant HRC 1702, and by Public Health Service grant AI 07137 from the National Institutes of Health.

We thank Chao-Ming Tsai for his assistance with the silver staining method and Marilyn R. Loeb for assistance with blot transfer technology.

LITERATURE CITED

1. **Allen, P. Z.** 1965. Specific immunological reactions of polysaccharides. Methods Carbohydr. Chem. **5**:232–246.
2. **Allen, P. Z., M. C. Connelly, and M. C. Apicella.** 1980. Interaction of lectins with *Neisseria gonorrhoeae*. Can. J. Microbiol. **26**:468–474.
3. **Apicella, M. A., and N. C. Gagliardi.** 1979. Antigenic heterogeneity of the non-serogroup antigen structure of *Neisseria gonorrhoeae* lipopolysaccharides. Infect. Immun. **26**:870–874.
4. **Avrameas, S., and T. Ternynck.** 1967. Biologically active water-insoluble protein polymers. I. Their use for isolation of antigens and antibodies. J. Biol. Chem. **242**:1651–1659.
5. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
6. **Catlin, B. W.** 1977. Nutritional requirements and auxotyping, p. 91–109. *In* R. B. Roberts (ed.), The gonococcus. John Wiley & Sons, Inc., New York.
7. **Connelly, M. C., D. C. Stein, F. E. Young, S. A. Morse, and P. Z. Allen.** 1981. Interaction with lectins and differential wheat germ agglutinin binding of pyocin 103-sensitive and -resistant *Neisseria gonorrhoeae*. J. Bacteriol. **148**:796–803.
8. **Engvall, E., and P. Perlmann.** 1972. Enzyme-linked immunoadsorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129–135.
9. **Goldman, R. C., and L. Leive.** 1980. Heterogeneity of antigenic side chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. Eur. J. Biochem. **107**:145–153.
10. **Guymon, L. F., M. Esser, and W. M. Shafer.** 1982. Pyocin-resistant lipopolysaccharide mutants of *Neisseria gonorrhoeae*: alterations in sensitivity to normal human serum and polymyxin B. Infect. Immun. **36**:541–547.
11. **Hurlburt, R. E., and I. M. Hurlbert.** 1977. Biological and physicochemical properties of the lipopolysaccharide of *Chromatin vinosum*. Infect. Immun. **16**:983–994.
12. **Kabat, E. A.** 1961. Experimental immunochemistry, p. 871. Charles C Thomas Publisher, Springfield, Ill.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lindberg, B., J. Longran, and D. A. Powell.** 1977. Structural studies on the specific type-14 pneumococcal polysaccharide. Carbohydr. Res. **58**:177–186.
15. **Morse, S. A., and M. A. Apicella.** 1982. Isolation of a lipopolysaccharide mutant of *Neisseria gonorrhoeae*: an analysis of the antigenic and biologic differences. J. Infect. Dis. **145**:206–216.
16. **Morse, S. A., B. J. Jones, and P. G. Lysko.** 1980. Pyocin inhibition of *Neisseria gonorrhoeae*: mechanism of action. Antimicrob. Agents Chemother. **18**:416–423.

17. **Pazur, J. H., A. Cepure, and J. A. Kane.** 1973. Glycans from streptococcal cell walls. J. Biol. Chem. **248**:279–284.
18. **Pazur, J. H., and L. S. Forsberg.** 1978. Determination of the sugar sequences and the glycosidic bond arrangements of immunogenic heteroglycans. Carbohydr. Res. **60**:167–178.
19. **Pavla, E. T., and P. H. Makela.** 1980. Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Eur. J. Biochem. **107**:137–143.
20. **Perry, M. B., V. Daoust, B. B. Diena, F. E. Ashton, and R. Wallace.** 1975. The lipopolysaccharides of *Neisseria gonorrhoeae* colony types 1 and 4. Can. J. Biochem. **53**:623–629.
21. **Stein, D. C., V. L. Clark, and F. E. Young.** 1983. Inhibition of active transport and macromolecular synthesis by pyocin 103 in *Neisseria gonorrhoeae*. Sex. Transm. Dis. **10**:7–13.
22. **Tsai, C.-M., R. Boykins, and C. E. Frasch.** 1983. Heterogeneity and variation among *Neisseria meningitidis* lipopolysaccharides. J. Bacteriol. **155**:498–504.
23. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.